

Comparative analysis of halophilic microbial populations at Lake Tyrrell, Australia**Alina Siu****Abstract**

The study of life at extreme environments helps us better understand the limits of where organisms can exist and how life adapts to survive. We are studying Lake Tyrrell, south-eastern Australia, to gain information on life that survives in a specific extreme environment: evaporitic environments. Possible applications of studying the lake are evolution, biotechnology, and an analog for possible conditions on Mars and primitive Earth. A comparative analysis was conducted on halophilic microorganisms found in planktonic samples and microorganisms found in salt at Lake Tyrrell. As water evaporates from the lake, salinity increases and salt crystals form. When the crystals form, water is trapped within the matrix, and microbes can be found in these fluid inclusions. This study compares microbial populations trapped within salt crystals with previously studied halophilic microbial populations present free-floating in the lake. The DNA from two microbial populations were sequenced: planktonic populations and salt populations. From this, 16S clone libraries and an Archaea and a Bacteria phylogenetic tree with both populations was created. By attempting to study what halophiles are present in the salts through the sequencing of DNA, we have taken another step towards discovering what is viable in these salt crystals and thus what life can possibly survive in different and possibly new environments.

Introduction

With recent innovations allowing scientists to study new places in the world in multiple ways, the narrow conditions that were once believed to be required for life are changing. We now can find life surviving in environments that were once thought as barren and uninhabitable. The study of “extreme environments”, environments with a physical extreme such as temperature, pressure, pH, or salinity (Rothschild, 2001) allow scientists to examine under what conditions life can exist and how. In addition, the study of organisms thriving at these locations builds on the biodiversity of the world and how ecosystems function as a whole. An extreme environment of interest to scientists is an evaporitic environment, an environment with high sodium, magnesium, and/or potassium salt deposits and ephemeral water. Over time, evaporitic environments leave evaporitic deposits, which are considered signatures of past presence of water (Wentworth, 2003). If found in rocks, evaporitic deposits contain evidence of past life and a fossilized record of the development of life on Earth (Wentworth, 2003). Therefore, the study of evaporitic environments has become an important one. In addition, there is evidence that environments similar to the evaporitic ones on Earth may exist on Mars and thus water may have been present at one time on the red planet (Benison, 2003). Therefore, the study of evaporitic environments can lead to many future discoveries and develop our knowledge of life on Earth.

Lake Tyrrell (Figure 1), in Victoria, Australia, is an evaporitic environment. The lake groundwater has low pH (2.5-4.0) (Lyons *et al*, 1992), while lake waters vary from saline to hypersaline (35-250 ppt), oxic to anoxic, and exhibit broad seasonal temperature change (7-30°C) (Macumber, 1992). A result of Lake Tyrrell’s evaporitic nature is the precipitation of salt crystals and the

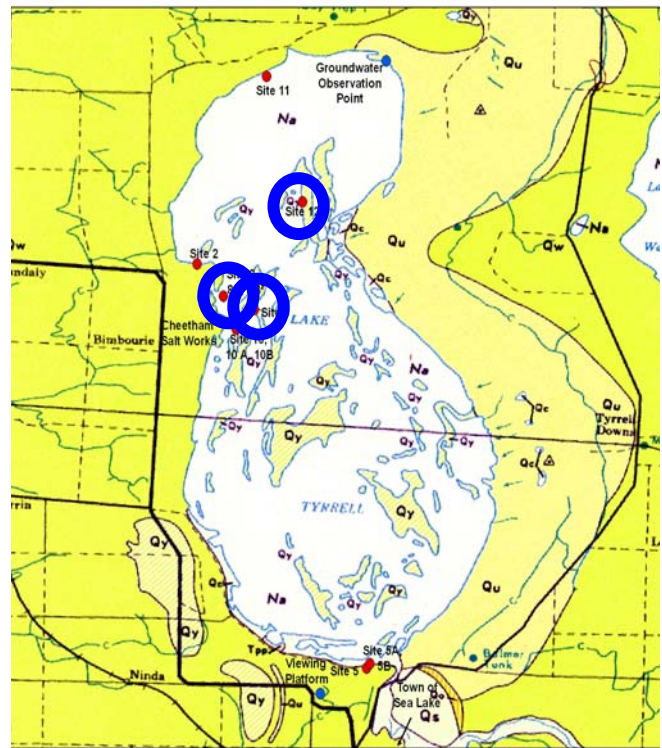


Figure 1. Map of Lake Tyrrell, Australia. Water sample sites 8, 9, and 12 are circled. Salt samples were only collected from site 9, the middle circle.

creation of an evaporitic mineral assemblage that leaves only the most salt tolerant (halophilic) members of the planktonic population. These members, in the process, are trapped in the fluid inclusions of the salt crystals, and are preserved. Moreover, preliminary studies (Banfield *et al*, unpublished) have determined this environment to be suitable for the studies of micro-organisms using standard methods. This study continues the molecular characterization of Lake Tyrrell's microbial community, focusing on a comparison between members of the planktonic community to those associated with fluid inclusions in the evaporitic mineral assemblage. This will determine whether there are halophiles in the community and which are the most halophilic members in order to better understand the history and possibilities of life.

Past Research The study of "extremophiles", life capable of surviving in extreme environments, allows scientists to understand many things about the survival of life not only on Earth but also on Mars (Rothschild, 2001). By studying extremophiles, the limits of life can be understood (Benison, 2003). The presence of evaporites, as in Lake Tyrrell, provides an extreme setting of how life evolves, as well as where signs of biological life would be preserved in space (Wentworth, 2003) and time. The study of Lake Tyrrell will greatly contribute to the knowledge of life in extreme environments.

It is possible for life to be preserved in the fluid trapped in the salt crystals in extreme environments (Goldstein, 2001). Pasteris (2006) showed that halite (sodium chloride) can accumulate and preserve organic material and allow for conditions in which microbes, trapped in the crystals, can survive. Nucleic acids are susceptible to degradation by UV radiation (Rothschild, 1990). But if trapped in fluid in halite, viable bacteria spores can survive for a long time (Kminek, 2003).

Microbes have been found in salt crystals in other studies and imply a mechanism of the preservation of life in evaporitic environments. Bacteria are capable of being included in the fluid trapped in halite (sodium chloride) in laboratory conditions (Adamski *et al*, 2006). In addition, salt crystals from multiple environments have been shown to harbor microbes: Vreeland (1998) found microbes from salt collected at waste isolation salt mine plant, Caton (2003) in the Great Salt Plains of Oklahoma, and Mormile (2003) in Death Valley, California. However, although there have been many findings of microbes in salt crystals, it is not a widely accepted idea. Controversies include the consistency of results and purity of samples (Vreeland,

1998). These discrepancies must be resolved, and more confirming studies conducted before the idea that microbe preservation in fluid inclusions of salt crystals is generally adopted.

Because the lake is saline (Macumber, 1992) and at times the evaporation from the lake exceeds precipitation (Macumber, 1982), salt crystals are left exposed and it is possible that most communities reliant on water disappear. This would result in only the most halophilic members left. These microbes, although maybe not active, are believed to be trapped in the remaining fluid inclusions of the salt crystal matrix (Fish, 2002; Mormile, 2003; Vreeland, 1997). This study addresses the planktonic community before water evaporates in Lake Tyrrell, and which members can survive in the salt crystals after evaporation.

Preliminary molecular data have been collected for the planktonic microbial community in the lake (Banfield *et al*, unpublished). 16S rRNA gene libraries have been constructed for the planktonic community, identifying both Archaea and Bacteria. The Archaeal genera detected are members of *Haloferax*, *Haloarcula*, *Halobacterium*, and *Halorubrum* in the family Halobacteriaceae (NCBI). *Halorubrum* was the dominant genus in the water. Samples also revealed the presence of the Bacterial genera *Salinispharea* and *Salinibacter*. However, these molecular analyses have been limited to the planktonic community, excluding the microbial population in the salt crystals.

Lake Tyrrell, as an evaporitic environment, leaves behind salt crystals when its water evaporates. This selects for the most halophilic members of the planktonic population and may lead to the preservation of these extremely halophilic community members within the fluid inclusions of the salt crystals. This study determines how microbes associated with the salt crystals is related to the planktonic community present in the overlying water; are they a subset of the planktonic population or a unique community? This project will help guide future investigations on other evaporitic environments as well as how planktonic populations relate to those preserved in the evaporites and salt.

Methods

Sample Collection The water samples for planktonic community analysis were previously collected from sites 8, 9, and 12 (circled, Figure 1) They were sampled by filtration and sequenced by members of the Banfield group. The salt samples were collected from Lake

Tyrrell, at site 9 (middle circle, Figure 1), in January 2007. These samples were collected and sealed in sterile plastic bags.

DNA Extraction A combination of procedures was used to extract DNA from the salt crystals. To remove humic material and possible contamination, the crystals were washed with a 2M brine solution in a sterilized strainer. The salt was then slowly dissolved with sterile water while maintaining the solution near saturation (Raddax *et al*, 2001) to a final volume of approximately 33ml. 15ml of the solution was then centrifuged at 9000g for 10 minutes and the supernatant was discarded. This process was repeated until all of the solution was gone. The DNA was then resuspended and the extraction was completed using Qiagen's DNAeasy Tissue Extraction kit, according to manufacture's protocol (Valencia, CA).

PCR amplification of the 16S DNA fragments The DNA was amplified using universal primers 23F (archaeal) , 27F (bacterial) and 1492R with an annealing temperature gradient from 43C to 60C. Products were pooled, confirmed via gel electrophoresis, and subsequent clean-up was performed using QIAquick PCR Purification kit (Valencia, CA).

Cloning of PCR products Products were ligated and transformed into *E. coli* using Invitrogen's TOPO TA kit (Carlsbad, CA). The positive colonies were selected and grown overnight in 96-well plates. Each individual well held a "clone" that had inserted into it a 16S DNA sequence from an individual microbe taken from the salt sample. Each clone represents one microbe from the salt or planktonic sample. Mini-preps and sequencing were performed by the UC Berkeley DNA Sequencing facility, using a Biomek robot running Agencourt protocols and an ABI 3730 prism Sanger sequencer, respectively.

Phylogenetic Analysis Partial 16S sequences (approximately 750 bp) were processed by removal of vector and trimming of poor quality sequences. BLAST searches within the NCBI database were done to identify known close relatives. Results were limited to halophilic species and identified with a matching percentage of at least 90%. Multiple alignments were created for archaeal and bacterial sequences using the Greengenes NAST aligner (DeSantis *et al*, 2006). These alignments were then uploaded into ARB and maximum likelihood trees were created (Stunk *et al*, 1996). Bootstrap values were calculated for approximately 100,000 tree samples.

Results

The microbial population associated in salt crystals was compared with the previously analyzed planktonic population. PCR products had the lengths (1.5-1.6kb) and concentrations that were expected of the primers used. There were low transformation concentrations to *E. coli* for archaeal DNA, but high concentrations for bacterial DNA.

The BLAST results from the NCBI database resulted in 8 unique bacterial sequences out of 63 clones, and 11 unique archaeal sequences out of 24 clones. Microbes from both planktonic and salt crystal samples are in the Archaea and Bacteria domain (Table 1). Two phylogenetic trees were created from these sequences, one for Archaea and one for Bacteria were created and are shown below (Figure 2 and 3, respectively).

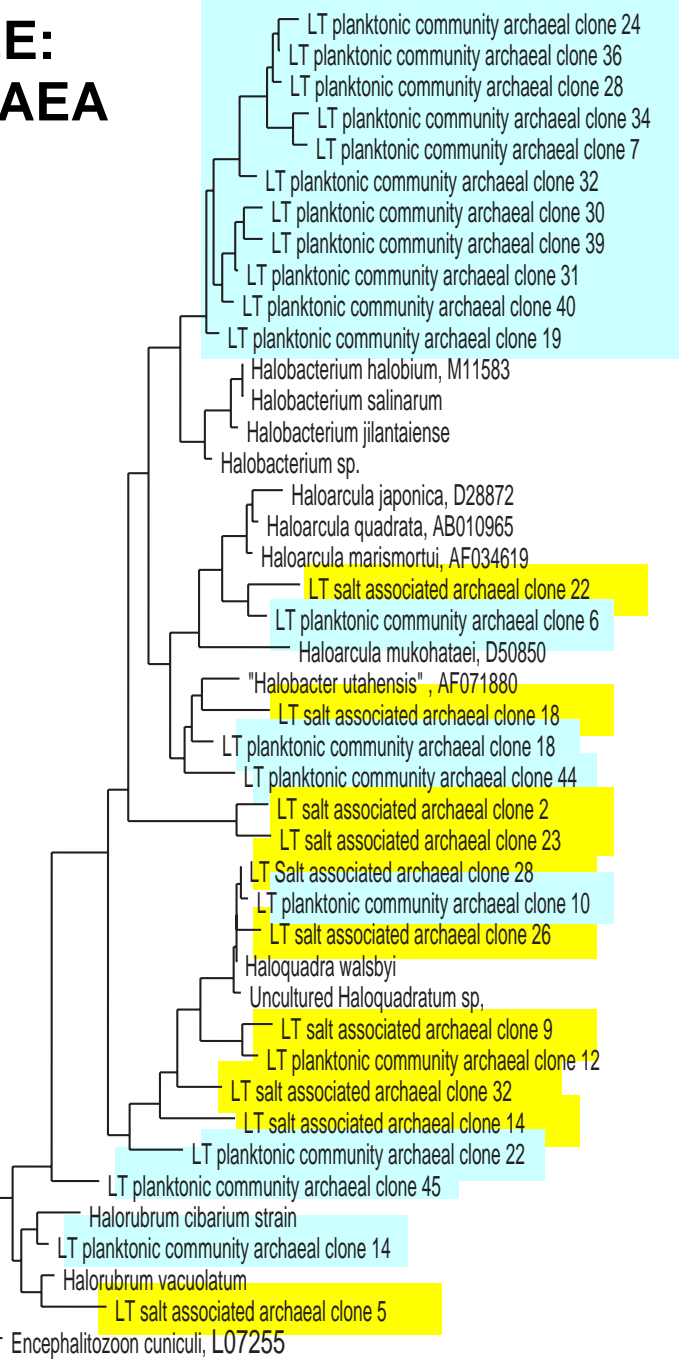
Eight haplotypes of salt crystal-extracted microbes (labeled “LT salt associated bacterial clone”) belonged to four unique clades within the Bacteria domain (Figure 2). Four haplotypes, differing by only a few basepairs, cluster together within the class Sphingobacteriales. Two haplotypes cluster within the class Alphaproteobacteria. The remaining two clades are represented by individual haplotypes in the classes Sphingobacteriales and Alphaproteobacteria. In each case, the sister clades are planktonic halophiles, except the clade with the two haplotypes in the class Alphaproteobacteria. That clade is sister to all of the planktonic communities, which belong to the class Gammaproteobacteria.

Name	From salt only	From planktonic only	From both
<i>Haloquadra</i> (13 haplotypes)	+		
<i>Halorubrum</i> (9 haplotypes)			+
<i>Haloferax</i>		+	
<i>Haloarcula</i> (2 haplotypes)			+
<i>Halobacterium</i>		+	
<i>Bacteriodetes</i> (31 haplotypes)	+		
<i>Salinibacter</i> (32 haplotypes)			+
<i>Salinispharea</i>		+	

Table 1. Microbes found in salt and planktonic samples.

TREE: ARCHAEA


from salt
 from water

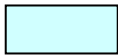


Genus:
 Halobacterium
 Haloarcul
 a
 Haloquadr
 a
 Halorubrum
 Family: Halobacteriaceae

Figure 2. Phylogenetic tree of the Archaea domain. Microbes from the salt crystals are labeled "LT salt associated archaeal clone #". Those from the planktonic samples are labeled "LT planktonic community archaea clone #".

TREE: BACTERIA

 from salt

 from water

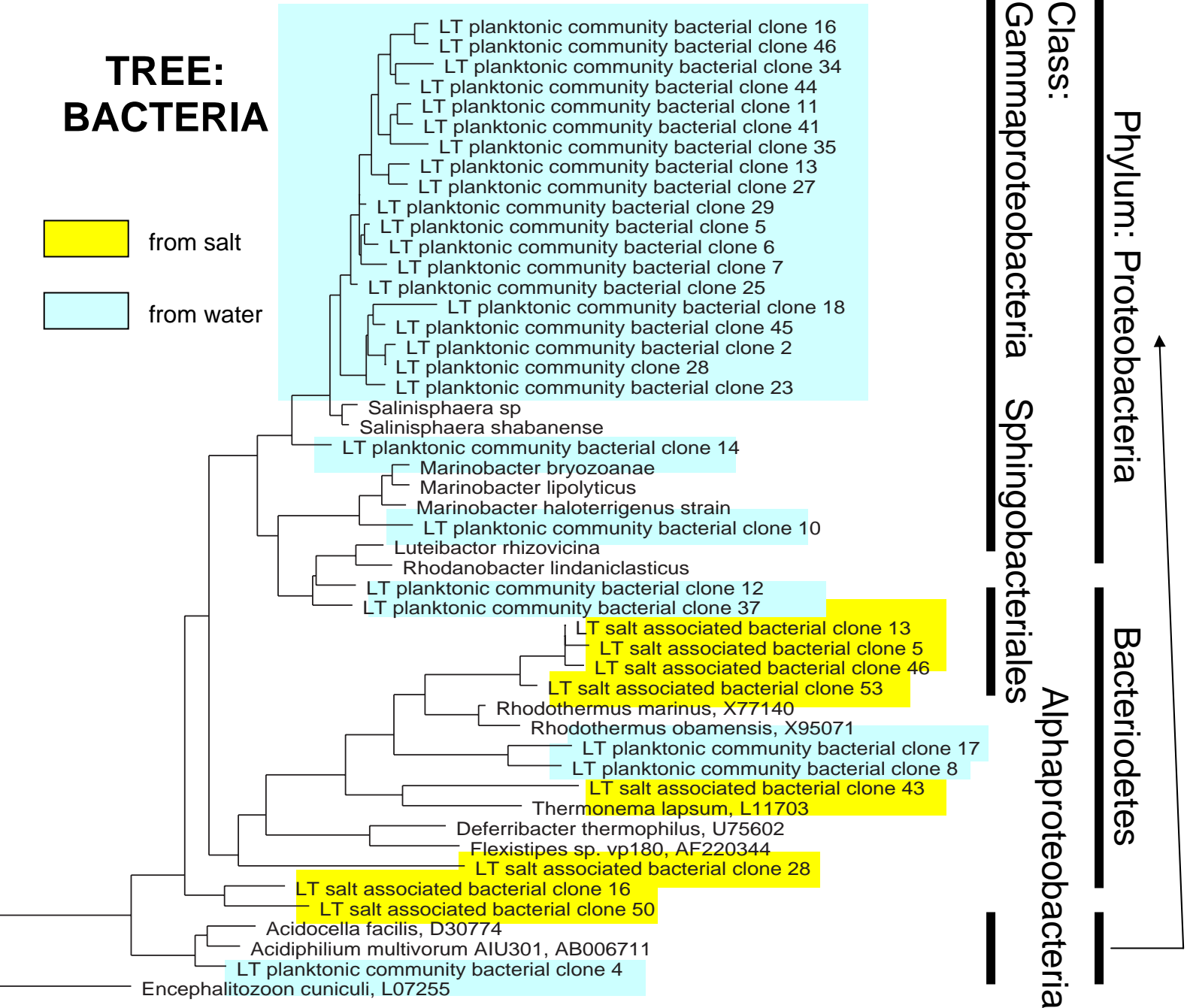


Figure 3. Phylogenetic tree of the Bacteria domain. Microbes from the salt crystals are labeled “LT salt associated bacteria clone #”. Those from the planktonic samples are labeled “LT planktonic community bacteria clone #”.

Eleven haplotypes of salt crystal-extracted microbes are scattered throughout the family Halobacteriaceae in the Archaea domain (Figure 3). The sister clades to each of these haplotypes are Lake Tyrrell planktonic halophiles, while the remaining clades are halophiles from other saline systems. Two clades each characterized by a single haplotype, belonged to the genus *Haloarcula*. There are also two clades, one with two haplotypes and one with six haplotypes, that belong to the genus *Haloquadra*. Finally, there is one clade with an individual haplotype that belongs to the genus *Halorubrum*.

Discussion

In general, the trees show relationships between the planktonic community and the salt crystal community. There is low biodiversity within the microbes that were found in the salt samples relative to the planktonic community. When looking at the trees, one needs to look at common ancestors and consider the different explanations to connections between the two populations. To construct the tree, reference microbes were taken from the National Council for Biotechnology Information (NCBI) website. There are branches at nodes where there would be salt clones at one clade and planktonic clones at another clade. This would be a point of interest to discuss survival in a salt crystal. One possible explanation is that at the node, the ability to survive in a salt crystal is gained. However, another explanation is that the ability to survive had previously been acquired, and that at the node of interest, the adaptation was lost. Alternatively, if the clones are closely related (as indicated by short branch lengths on the tree), the microbes may actually be the same species. These explanations are further discussed below for the Bacteria and Archaea tree.

In the Bacteria tree, the clones from the salt crystals appear separate from the clones in the planktonic community, yet still related to them. The salt bacteria haplotypes #5, 13, 46, and 53 form a single clade and are sister to the planktonic bacteria haplotypes #8 and 17. Salt bacteria clone #43 is also shown be a sister to the planktonic bacteria haplotypes #17 and 8. So it is possible that there were two adaptations in this section of the tree that allowed for microbes to be in salt crystals, one adaptation as shown in the split between the planktonic bacteria clones and salt bacteria clone #43, and the other adaptation shown in the split between the planktonic bacteria clones and salt bacteria clones #13, 5, 46, and 53. However, an alternate explanation is that there was one adaptation to living in salt crystals that includes salt bacteria clones # 13, 5,

46, 53, and 43, and that the adaptation was lost in planktonic bacteria clones #17 and 8. At first glance, both scenarios are likely explanations and the calculation of which is more probable is a necessary step in the future.

In the Archaea tree, the clones from the salt crystals are closely integrated with those from the planktonic community, and almost all the salt haplotypes have a close sister planktonic haplotype. For example, salt archaeal clone #22 is very similar to planktonic archaea clone #6, salt archaea clone #18 is very similar to planktonic archaea clones #18 and #44, and salt archaea clones #28 and #26 are very similar to planktonic archaea clone #10. There is a group of salt archaea clones (clones #9, 12, 32, and 14), but their short branch lengths indicate there is not a large difference in the DNA compared to the closest planktonic archaea clone (clone #22). Again, there are a few explanations to account for these close associations. One is that there are many adaptations that occurred to allow microbes to be in salt crystals. Another is that there were only a few adaptations and at different points, the adaptations were lost. The final possible explanation is that each salt archaea clone is actually the same species as its similar planktonic archaea clone. This may be a likely explanation in the archaea case because the two communities have similar microbes and because the branches on the tree are only a few basepairs long, showing only a few nucleotide differences in the DNA. Again, the probability of each scenario needs to be calculated in a future study.

There are a few limitations to my data that need to be conceded. Firstly, the planktonic samples and the salt samples were not all collected at the same place and time. Planktonic samples were collected at site 8, 9, and 12 (Figure 1) while salt samples were only collected at site 9. Though we do not anticipate this to be a major hindrance, there may be fine details that have been overlooked. Certain organisms found might be specific to a certain environment in the lake for multiple reasons. Also, this project only sequenced the DNA extracted from the salt and planktonic samples. This proves a presence of organisms, but cannot claim anything about the activity of the organisms. To conclude that the microbes in the salt and in the water were alive and active (rather than simply preserved by the high saline environment), RNA would have to be extracted. In addition, one needs to critically look at the accuracy of the tree and run more models with different parameters to verify the correct tree. The evolutionary model used in ARB to create the trees had strict parameters, but more verification is needed. Finally, we cannot be positive that the DNA is only from the inside of the salt crystals. Though our methods tried to

wash off anything from the outside of the crystals by dissolving the outer layer, DNA might have adhered again to the salt.

The results of this project proves that microbes can be preserved in halophilic environments. It also demonstrates that halophiles associated with the salt crystals are related to the planktonic community, and that the salt crystal community is actually a subset of the planktonic community. Each unique clade characterized by a salt haplotype has a sister clade with a planktonic haplotype. For Archaea domain, there are microbes from the same genus in the salt and planktonic community (Table 1) and the similar salt and planktonic microbes are only a few basepairs different, showing that they may actually be the same species. This may show that the salt crystal-extracted archaea microbes are representative of the planktonic archaeal community. However, in the Bacteria domain the salt microbes are distinctly in a different clade, and some even in a different class, than the planktonic microbes. However, to initially be trapped in the salt crystals, the microbe must have existed in the water. Therefore, it is probable that the salt halophiles were from the planktonic community, but not abundant, so it was not found in the water samples. Instead, they are the only ones to be preserved by salt crystals, and so the salt crystal-extract bacteria microbes are only a part of the planktonic bacterial community and not representative of it. When studying fossilized microbes in evaporitic deposits, one then must keep this in mind; they may be looking at a specific subset of the whole community, or they may be collecting a community representative of the whole. This report suggests that it depends on the domain; in the Archaea domain, the microbes found associated with the salt, and thus in the evaporitic deposits, are representative of the planktonic archaea community, while in the Bacteria domain, the microbes found associated with the salt are only a section of the planktonic bacteria community. However, further, more extensive studies in other evaporitic environments must be conducted to confirm this hypothesis.

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